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The Envelope Glycoprotein of an Amphotropic Murine Retrovirus Binds Specifically to the Cellular Receptor/Phosphate Transporter of Susceptible Species

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A rat cDNA (rRam-1), which was cloned on the basis that it enables Chinese hamster ovary (CHO) cells to be infected by amphotropic host range murine retroviruses, was recently found to encode a widely expressed Na^+-phosphate symporter (M. P. Kavanaugh, D. G. Miller, W. Zhang, W. Law, S. L. Kozak, D. Kabat, and A. D. Miller, Proc. Natl. Acad. Sci. USA 91:7071–7075, 1994). CHO cells express the hamster homolog of Ram-1 but are resistant to amphotropic retroviruses. Although the amphotropic envelope glycoprotein gp70 bound weakly onto control CHO cells, CHO/rRam-1 cells had novel high-affinity binding sites, and the resulting strongly adsorbed gp70 was only slowly removed from cell surfaces, with a half-life of greater than 6 h. CHO/rRam-1 cells were also specifically and efficiently killed by exposure to amphotropic gp70 followed by antiserum to gp70 in the presence of complement. Infection with an appropriately pseudotyped form of amphotropic retrovirus 4070A did not perturb control CHO cells or inhibit their phosphate transport. In contrast, 4070A infection of CHO/rRam-1 cells caused major alterations including cell-cell fusions, a specific 40% down-modulation of the rRam-1 component of phosphate transport, and complete interference to superinfection by amphotropic viruses. The 4070A virus-infected CHO/rRam-1 cells retained a substantial cell surface pool of rRam-1 that functioned as a phosphate transporter but not as a viral receptor. We conclude that amphotropic gp70 binds more strongly to rRam-1 than to the homologous hamster protein and that this stable attachment is necessary for infection, interference, membrane fusion, and pathogenesis.

A cDNA that encodes the putative cell surface receptor for amphotropic host range murine leukemia viruses (MuLVs) was independently cloned from rats on the basis that it conferred susceptibility to infection on Chinese hamster ovary (CHO) cells (24) and from humans on the basis of partial homology to the cell surface receptor (Glvr-1) for Gibbon ape leukemia viruses (37) and for feline leukemia viruses of subgroup B (36). The recombinant rat amphotropic receptor will be abbreviated rRam-1, in distinction to homologous Ram-1 proteins of other species. Weak homology of Glvr-1 to a fungal phosphate transporter (15) had suggested that Glvr-1 might be a member of a possibly diverse family of transporters, and this hypothesis was recently confirmed by direct evidence that both Ram-1 and Glvr-1 are major Na^+-phosphate symporters in many cells and tissues (17, 28).

Because infections by some viruses, including retroviruses, may require species- or cell-specific membrane-associated accessory factors or coreceptors (7, 12, 13, 20, 38, 41), it cannot be inferred that Ram-1 or Glvr-1 directly mediates virus adsorption onto cells. Amphotropic MuLVs infect almost all mammalian species, and the mechanism for CHO cell resistance is unclear. Previous evidence suggested that amphotropic MuLVs bind relatively poorly onto CHO cells (11, 17), but this could be due to low cell surface expression of the hamster homolog of Ram-1, due to mutations in the hamster protein that reduce its affinity for virus, or due to a coreceptor deficiency. Treatment of CHO cells with inhibitors of asparagine-linked glycosylation cause the cells to become susceptible to amphotropic MuLV infections (25, 26, 43), and lectin-resistant CHO cell mutants that synthesize glycoproteins with truncated oligosaccharides are also susceptible (26). Additionally, hamster cells secrete a factor that inhibits their infection by amphotropic MuLVs (25, 26). This evidence has implied that hamster cells are not absolutely resistant to amphotropic MuLVs and that cell surface molecules needed for their infection may be masked by glycosylation or by a secreted factor. RNA blot analysis has shown that CHO cells and susceptible NIH 3T3 mouse fibroblasts contain similar amounts of Ram-1-related mRNAs (24).

Interactions of retroviruses with cell surface receptors have been implicated not only in virus entry into cells but also in interference to superinfection, immunosuppression, mitogenesis, and cytopathology (9, 14, 27, 29, 30, 32–34, 40). Amphotropic MuLV infection of some cells causes substantial cell killing and cell-cell fusion (e.g., see below). Recently, we found that expression of an amphotropic *env* gene but not an ecotropic *env* gene caused an approximately 40 to 50% reduction in phosphate transport by mouse NIH 3T3 fibroblasts (17), suggesting that the mouse homolog of Ram-1 might be a major phosphate transporter in these cells and that its activity or expression might be partially or fully blocked by amphotropic *env* glycoproteins. However, that study was ambiguous because NIH 3T3 fibroblasts express multiple phosphate transporters and because there are no negative control mouse fibroblasts that lack amphotropic receptors. The previous results would be compatible with a nonspecific effect of amphotropic *env* glycoproteins on membrane potential or on intracellular Na^+ or K^+ concentrations. For all of these reasons, we have used CHO cells to more thoroughly analyze interactions of rRam-1 with amphotropic *env* glycoproteins. Our results suggest that the viral glycoprotein interacts more strongly with rRam-1 than

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with the homologous hamster protein. Interference to superinfection is achieved without eliminating rRam-1 from cell surfaces.

MATERIALS AND METHODS

Cell clones. Normal CHO fibroblasts are resistant to infection by both amphotropic and ecotropic murine retroviruses (24, 37). However, for the studies reported here we used a clone of CHO cells (CERD-Cl9 cells) that expresses recombinant ecotropic receptors (39). rRam-1 cDNA was subcloned into the expression site of the retroviral vector pSFF (1). The pSFF-rRam-1 plasmid was transfected into a coculture of ψ2 ecotropic packaging cells (21) and PA12 amphotropic packaging cells (23) for ping-pong amplification of helper-free SFF-rRam-1 virions (1, 19). These virions were used to infect the CERD-Cl9 line of CHO cells. Cell clones from the latter culture were isolated by limiting dilution, and clones were screened for rRam-1 expression by testing for susceptibility to amphotropic virus infection. The selected clones, called CHO/rRam-1, lacked *env* glycoprotein expression, and they all bound amphotropic gp70 (see below).

To express amphotropic *env* glycoproteins in CERD-Cl9 and CHO/rRam-1 cells, two viral preparations were used. First, to infect CERD-Cl9 cells that lack rRam-1, the 4070A virus was first used to infect ψ2 ecotropic packaging cells (these cells have functional amphotropic receptors) (1, 21). The 4070A virus released from these cells, which was a pseudotype with ecotropic *env* glycoproteins, was then used to infect the CERD-Cl9 clone of CHO cells. For comparative studies, these CHO and CHO/rRam-1 cell clones were infected in parallel cultures, using this pseudotyped form of 4070A virus. Second, replication-competent 4070A virus was used to infect CHO/rRam-1. As expected, all of these 4070A-infected cells expressed amphotropic *env* glycoproteins and remained susceptible to infection with ecotropic host range viruses.

CHO cells and their derivatives were grown in the α modification of minimum essential medium supplemented with 10% fetal bovine serum. All other cells were maintained in Dulbecco's modified Eagle medium with 10% fetal bovine serum.

Binding assays. Amphotropic 4070A virus was obtained by centrifugation of 1.5 liters of medium from a culture of infected mouse BALB/c 3T3 fibroblasts (11). The gp70 was then released from the virions by freeze-thawing and was recovered in the supernatants after sedimentation of the residual viral particles as described elsewhere (11, 35). The amphotropic gp70 preparations (aliquots were adjusted to 4 mg of total protein per ml and were frozen at −80°C) maintained their receptor-binding activities through repetitive freeze-thaw cycles. For analyses, gp70 preparations were incubated with cell cultures for 2 h at 37°C in amounts specified for each experiment. After removal of unbound gp70, the cells were rinsed with fresh medium and were then incubated for 1 h at 37°C with a 1:200 dilution of a goat antiserum made to Friend ecotropic viral gp70 that cross-reacts strongly with amphotropic gp70 (10, 19). After the cells were rinsed with fresh medium, the adsorbed gp70-antibody complexes were detected by three methods. In one method, cells were incubated with fluorescein-labeled rabbit anti-goat immunoglobulin G (1:200 dilution in culture medium; Organon Teknika, Durham, N.C.) for 1 h at 37°C and then rinsed three times for 5 min each with fresh medium. The cells were then fixed with ice-cold methanol, mounted in 10% glycerol in phosphate-buffered saline, and examined by immunofluorescence microscopy. A second detection method involved incubation with 0.4 μCi of [^{125}I]protein A (DuPont NEN, Wilmington, Del.) per ml for 1 h, rinsing away the unbound radioactivity, and counting the cells in a gamma counter as described elsewhere (39). The third detection method involved incubation with a 1:12 dilution of rabbit complement (GIBCO BRL, Grand Island, N.Y.) and measuring the release of radioactive proteins from the cells as previously described for other cell surface antigens (11). In this case, the cells were prelabeled by incorporation of L-[^{35}S]methionine (Amersham, Arlington Heights, Ill.) at 50 μCi/ml (1,000 Ci/mmol) for 2 h followed by a 2-h chase in fresh complete medium prior to incubation with the other reagents.

Other methods. All other methods have been previously described. These include assays for cellular uptake of different concentrations of $^{32}P_i$ (18), least-squares fitting of the resulting curves to a Michaelis-Menten equation by a computer program (Kaleidograph) (38), immunoprecipitation, and Western blot (immunoblot) methods for distinguishing amphotropic and ecotropic *env* glycoproteins (10, 19). Quantitative virus infection assays used helper-free retrovirions with amphotropic envelope glycoproteins that encode human growth hormone (19, 39). In this assay, the infected cells with growth hormone are detected by immunofluorescence, and the multiplicity of infection is then calculated from the fraction of infected cells. Protein concentrations were measured by the Coomassie blue dye method (Bio-Rad, Richmond, Calif.).

RESULTS

CHO/rRam-1 cells have novel high-affinity binding sites for amphotropic gp70 that are absent from control CHO cells. To study the role of rRam-1 in virus binding, we incubated CHO cells (clone CERD-Cl9) and its CHO/rRam-1 derivative for 2 h with amphotropic gp70 and subsequently for 1 h with a specific antiserum to gp70 and then for 1 h with a fluorescein-conjugated second antibody. CHO/rRam-1 cells became highly fluorescent in this gp70-dependent reaction, whereas control CHO cells bound relatively little gp70 (Fig. 1).

Additional evidence for gp70 binding was obtained by using [^{125}I]protein A instead of fluorescein-labeled second antibody and by titrating this radioactivity assay with different concentrations of the gp70 preparation (Fig. 2). The antiserum to gp70 and [^{125}I]protein A were present in large excesses. Although control CHO cells bound gp70 with relatively low affinity, CHO/rRam-1 cells bound substantially larger amounts in a manner that saturated as the gp70 concentration increased. Thus, CHO/rRam-1 cells have a class of relatively high affinity sites for gp70 that are absent from control CHO cells.

When incubated with gp70 and then with antibody to gp70 and with complement, CHO/rRam-1 cells were rapidly and specifically killed (Fig. 3). The cells, which had been labeled by incorporation of L-[^{35}S]methionine, lifted from the substratum, developed pycnotic nuclei, and leaked their radioactive proteins into the medium. Control CHO cells completely resisted this gp70-dependent killing, although the viable cells slowly degraded their proteins and released some radioactivity into the medium.

The foregoing assays for gp70 binding each involved prolonged sequential incubations of viable cells at 37°C with several reagents, and the results suggested that adsorbed gp70 must remain accessible to extracellular reagents for at least several hours. To directly study the rate of gp70 removal from CHO/rRam-1 cell surfaces, we allowed gp70 to adsorb for 2 h and then rinsed the cells and incubated them in fresh medium for various times prior to additions of antibody to gp70 and of [^{125}I]protein A. The CHO/rRam-1 cells lost gp70 from their surfaces with a half-life of greater than 6 h (Fig. 4).

Amphotropic envelope glycoprotein synthesis partially down-modulates rRam-1 transporter expression in CHO cells but has no effect on endogenous hamster transporters. In agreement with recent evidence for Ram-1-mediated phosphate transport in *Xenopus* oocytes and rat 208F fibroblasts (17), expression of rRam-1 in our CERD-Cl9 clone of CHO cells resulted in elevated phosphate transport that behaved in accordance with Michaelis-Menten kinetics (Fig. 5). The K_m for the rRam-1 component of transport in these cells was 270 ± 70 μM. Although a saturating concentration of gp70 as estimated from Fig. 2 had no effect on phosphate transport activity of control CHO cells, this gp70 caused a slight but reproducible 10 to 20% inhibition in V_{max} for transport by CHO/rRam-1 cells (Fig. 6A). Moreover, efficient infection of CHO/rRam-1 cells with amphotropic MuLV 4070A (levels of infection were above 99% as seen by immunofluorescence microscopy or by interference [see below]) caused a much stronger decrease in phosphate transport activity (Fig. 6A). To determine whether this infection-mediated decrease in phosphate transport was specific for the rRam-1 transporter, we infected our control CERD-Cl9 clone of CHO cells with an appropriately pseudotyped form of the amphotropic MuLV 4070A (see Materials and Methods). The resulting cells, which contain 4070A virus but lack rRam-1, had the same amount of phosphate transporter as uninfected control CHO cells (Fig. 6B). In contrast, infection of CHO/rRam-1 cells with the 4070A virus reproducibly caused a substantial and highly significant (n = 6) down-modulation in phosphate transport. Thus, down-modulation caused by 4070A virus infection is specific for the rat protein rRam-1. Similarly, the weaker inhi-

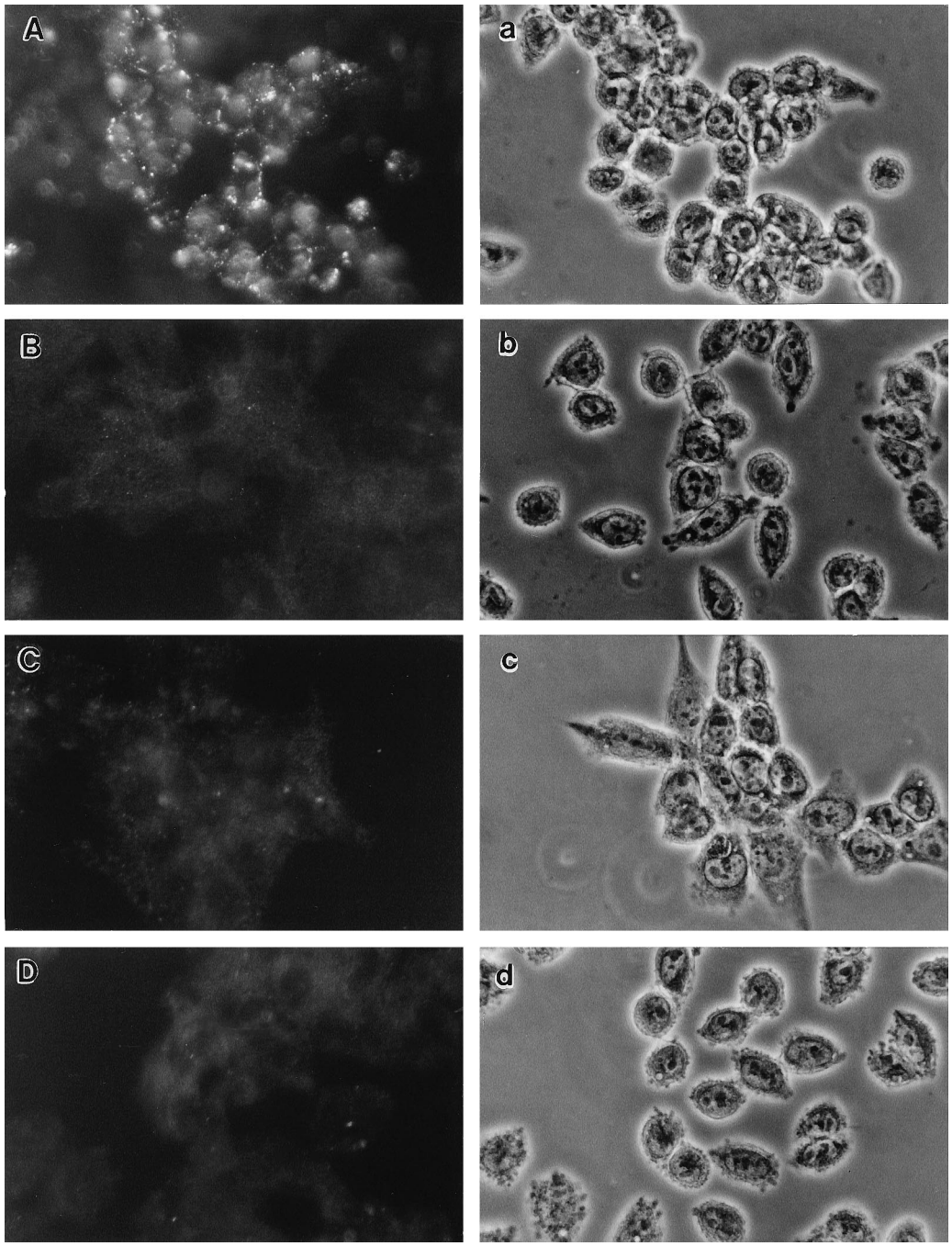

FIG. 1. Immunofluorescence assay for binding of amphotropic envelope glycoprotein gp70 onto cells. Cells were seeded in an eight-well chamber slide (5×10^4 cells per well) the day before. Cells were incubated for 2 h at 37°C with or without of the 4070A gp70 preparation (0.4 mg of protein per ml) followed by sequential 1-h incubations with goat antiserum to gp70 (1:200) and then with fluorescein-conjugated rabbit anti-goat immunoglobulin G (1:200). The cultures were fixed and mounted for fluorescence microscopy. The photographs are gp70-specific immunofluorescence (left) and the corresponding cells as seen by phase-contrast microscopy (right). The panels show CHO/rRam-1 clone 13 incubated with (A and a) or without (B and b) 4070A gp70 and control CHO cells incubated with (C and c) or without (D and d) 4070A gp70.

bition caused by extracellular gp70 also appears to be specific to rRam-1.

Interference to amphotropic MuLV superinfection. Consistent with previous studies using other cells and retroviruses (3, 6, 9, 36, 40), infection of CHO/rRam-1 or mouse BALB/c 3T3 fibroblasts with amphotropic MuLV 4070A caused complete (greater than 400-fold) resistance to superinfection with a helper-free amphotropic host range virus (SFF-hGH) (19) that encodes human growth hormone (Table 1). This efficient interference to superinfection could not be explained simply by the partial down-modulation of cell surface rRam-1 (e.g., as in Fig. 6B), because CHO/rRam-1 cell clones with relatively little rRam-1 are efficiently infected in the conditions of our studies (Table 2). For example, CHO/rRam-1 clone 40 cells express

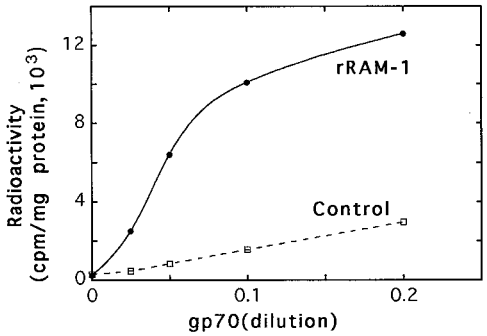

FIG. 2. Radioactive assay for binding of amphotropic gp70 onto CHO cells that contain or lack rRam-1. Cells expressing or lacking rRam-1 were seeded (2×10^5 cells per 2 cm^2) in wells of a 24-well culture plate the day before the assay. Wells were incubated with 0.25 ml of culture medium that contained various dilutions of the stock 4070A gp70 preparation for 2 h at 37°C. After being rinsed with complete medium, the cultures were incubated with goat antiserum to gp70 (1:200) for 1 h and then in medium with [^{125}I]protein A (0.4 μCi/ml) in a volume of 0.25 ml per well for 1 h at 37°C. After being washed, the cells were solubilized in 0.5 ml of 0.1 N NaOH. A small aliquot was used to determine protein concentration, and the samples were then counted in a gamma counter. Each datum point is the mean of replicate assays ($n = 2$). The data are unsuitable for estimation of binding constants because the gp70 preparations were impure and contained an unknown proportion of active molecules.

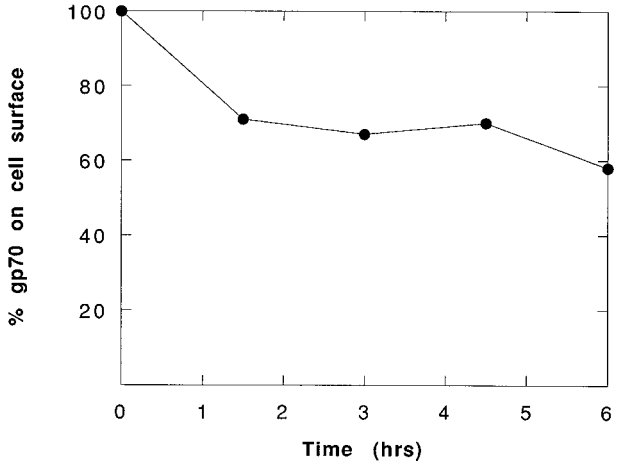

FIG. 4. Slow rate of removal of adsorbed gp70 from surfaces of CHO/rRam-1 cells. 4070A gp70 was adsorbed onto CHO/rRam-1 cells for 2 h at 37°C at a 1:10 dilution. The cells were then rinsed thoroughly and incubated in fresh complete culture medium for various times prior to incubation for 1 h with antiserum to gp70 and then for 1 h with [^{125}I]protein A as described in the legend to Fig. 2. Each datum point is the mean of replicate assays ($n = 2$).

only about 18% as much rRam-1 as clone 13 or clone 54 cells but are infected with approximately the same efficiency.

Cytopathic effects of amphotropic MuLV 4070A. The CHO cell clone (clone CERD-Cl9) was ideal for this project because it contains receptors for ecotropic mouse retroviruses and could consequently be easily infected with helper-free virions that encode rRam-1 (see Materials and Methods). Similarly, by passing amphotropic MuLV 4070A through ψ2 ecotropic retroviral packaging cells, we obtained a pseudotyped 4070A virus that was highly infectious for these CHO and CHO/rRam-1 cells, thereby enabling controlled comparisons.

An unexpected result of amphotropic 4070A virus infections was the initial extensive cytopathology and cell-cell fusions that resulted in the CHO/rRam-1 cultures (e.g., compare the infected and uninfected cultures in Fig. 7A and B, respectively). Approximately 40% of the nuclei in the infected CHO/rRam-1 culture occurred in syncytia. In contrast, as substantiated below, the infected CHO cells were resistant to this syncytium formation (see below). Moreover, infection with the 4070A virus caused a substantial selective reduction in growth rate of

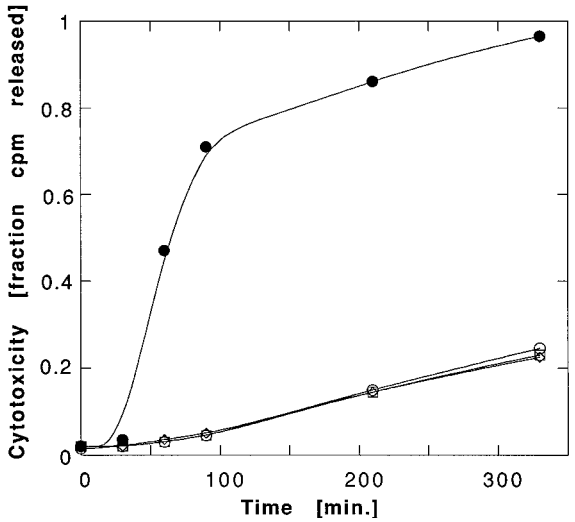

FIG. 3. Cytotoxic assay for amphotropic gp70 binding to CHO cells that lack or contain rRam-1. Cells, which had been seeded (2×10^5 cells per 25 cm^2) in tissue culture flasks 2 days before the assay, were labeled for 2 h with L-[^{35}S]methionine. The cultures were washed, incubated in complete medium for 2 h at 37°C, and then incubated for another 2 h in 2 ml of complete medium with or without a 0.2-ml supplement of the 4070A gp70 preparation. All cultures were washed and incubated with goat antiserum to gp70 (1:200) for 30 min prior to supplementation with a 1:12 dilution of rabbit complement. Duplicate aliquots (20 μl) of the media were removed for radioactive assays at the times indicated. At the end of the time course, the cultures were solubilized in 0.2% sodium dodecyl sulfate, and aliquots were assayed for radioactivity. The cells were CHO/rRam-1 incubated with gp70 (solid circles), CHO/rRam-1 (open circles), CHO incubated with gp70 (open squares), and CHO (open triangles). Each datum point is the mean of replicate assays ($n = 2$).

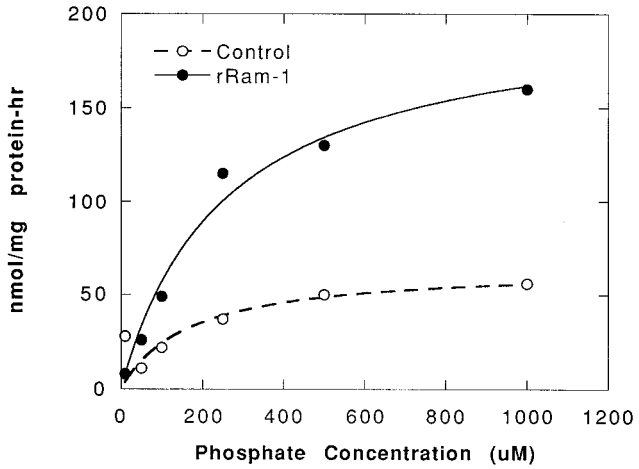

FIG. 5. Phosphate transport activities of control CHO cells and of CHO cells that express rRam-1. The initial rates of $^{32}P_i$ uptake are plotted as a function of total P_i concentration. The lines were drawn by least-squares computer fit to the Michaelis-Menten equation (Kaleidograph program) as previously described (38). The K_m and V_{max} values attributable to rRam-1 were 270 ± 70 μM and 130 ± 10 nmol/mg of protein per h, respectively. These values were determined from a plot in which the control CHO values were subtracted from the data for the CHO/rRam-1 cells. Each datum point is the mean of replicate assays ($n = 2$).

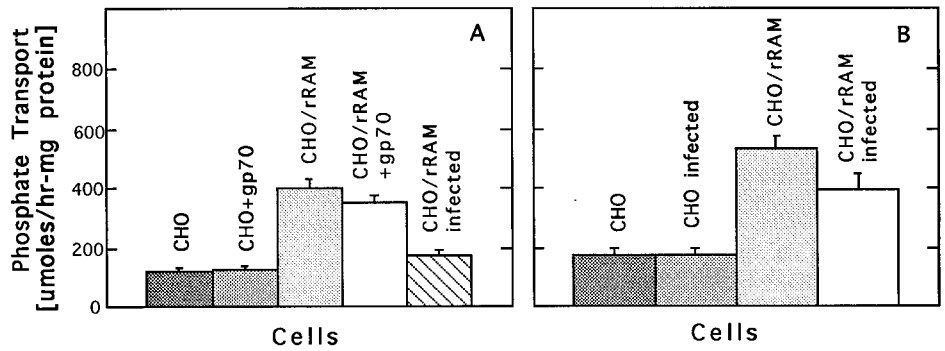

FIG. 6. Effects of extracellular amphotropic gp70 adsorption and of intracellular amphotropic *env* gene expression on the V_{max} for phosphate transport of CHO and CHO/rRam-1 cells. (A) Comparison of phosphate transport of CHO cells with and without 4070A gp70 and CHO/rRam-1 cells with and without 4070A gp70 ($n = 4$). The rightmost bar shows phosphate transport of CHO/rRam-1 cells that had been infected with the amphotropic virus 4070A 21 days prior to the phosphate transport analysis. (B) CHO and CHO/rRam-1 cells uninfected and infected with the 4070A virus ($n = 6$). The CHO cells lacking rRam-1 were infected by using an ecotropic pseudotype of the 4070A virus. The CHO/rRam-1 cells were infected and maintained in cultures for 33 days prior to phosphate transport analysis, and they had morphologically recovered from cytopathic effects of the 4070A virus infection. The error bars represent standard deviations. The down-modulation of transport caused by 4070A virus infection of CHO/rRam-1 cells is significant to the 95% confidence level for both panels. The reduction in transport of CHO/rRam-1 cells caused by extracellular gp70 is not significant to this level of confidence for the data in panel A. However, similar indications of specific rRam-1 inhibition by extracellular gp70 were seen in all of our independent assays.

the CHO/rRam-1 cells. This difference in effect of 4070A virus on CHO and CHO/rRam-1 cells was not due to a difference in efficiency of infecting these cultures. As seen by immunofluorescence microscopy or Western blotting for viral antigens, the CHO and CHO/rRam-1 cells were equally infected by the pseudotyped 4070A virus.

We observed that the cell-cell fusions and retarded growth rate described above gradually diminished after several weeks in the 4070A virus-infected CHO/rRam-1 cultures. Our interpretation is that the cell-cell fusion resulted from interaction of virus-infected cells with uninfected CHO/rRam-1 cells and that the fusion ceased after the virus had spread into all cells in the culture. Recovery then ensued when the polykaryons and polyploid cells were overgrown. The virus-infected cultures used for Fig. 6B and in Table 1 had all recovered from the initial cytopathic phase of the infection process as seen by microscopic inspection.

We verified several predictions of the cell-cell fusion model proposed above. Specifically, the 4070A virus-infected CHO cells and the infected CHO/rRam-1 cells that had undergone cytopathic recovery fused extensively when they were mixed 1:1 with uninfected CHO/rRam-1 cells but not when they were mixed with uninfected CHO cells. For example, Fig. 7 shows the consequences of mixing uninfected CHO/rRam-1 (Fig. 7C) or CHO (Fig. 7D) with the 4070A-infected CHO cells. In addition, we labeled the cellular DNAs by incorporation of bromodeoxyuridine and then used a fluorescent antibody to detect the labeled nuclei by microscopy (3). This analysis clearly showed that the polykaryons were syncytia that formed by fusion of bromodeoxyuridine-labeled 4070A virus-infected cells with unlabeled uninfected CHO/rRam-1 cells.

We also attempted to determine whether the presence of

TABLE 1. Interference to amphotropic retroviral superinfections

Cell line[a]	Infection efficiency[b] (MOI)
CHO/rRam-1	0.080 ± 0.009
CHO/rRam-1/MuLV 4070A	<0.0002
Mouse BALB	0.30 ± 0.033
Mouse BALB/MuLV 4070A	<0.0002
CHO	<0.0002
CHO/MuLV 4070A	<0.0002

[a] The cell lines, which were uninfected or infected with amphotropic MuLV 4070A prior to analysis, included CHO/Ram-1 (clone 13), mouse BALB/c 3T3 fibroblasts, and control CHO cells.

[b] Measured as described previously (39), using an amphotropic helper-free pseudotype of SFF-hGH, a virus that encodes human growth hormone. Infected cells were detected 48 h after infection by growth hormone-specific immunofluorescence (19). Medium containing virus (0.5 ml/2 cm^2) was incubated with cells in a 24-well culture plate. Because virus was in excess and was only partially adsorbed during incubation with the cell cultures, infection efficiency is a function of virus concentration and of cell susceptibility (16, 39). Multiplicity of infection (MOI) was calculated from the fraction (P_0) of cells that lacked growth hormone, using the equation MOI = $-\log P_0/0.44$ (19, 39). MOI differs from the fraction of growth hormone-positive cells by correcting for the fact that cells in highly infected cultures are often multiply infected. Errors are standard deviations based on counting statistics. For CHO/rRam-1 (clone 13) cells, 78 cells were positive among 995 counted; for BALB/c 3T3 fibroblasts, 75 were positive among 290 counted. For each of the other cultures, approximately 5,000 cells were scanned without any positives being detected.

TABLE 2. CHO/rRam-1 clones with different quantities of rRam-1 are equally susceptible to amphotropic virus infection

Cell clone[a]	rRam-1 level ([^{125}I]protein A binding in cpm/mg of protein)[b]	Infection efficiency (MOI)[c]
CHO	0 ± 240	0.004 ± 0.002
CHO/rRam-1		
Clone 13	7,100 ± 1,600	0.79 ± 0.055
Clone 54	6,900 ± 420	0.49 ± 0.034
Clone 38	3,050 ± 640	0.52 ± 0.044
Clone 19	2,850 ± 490	0.52 ± 0.038
Clone 40	1,450 ± 350	0.50 ± 0.036

[a] The CHO cell clone CERD-Cl9 was infected with helper-free ecotropic pseudotype virus SFF-rRam-1 to obtain a population of CHO/rRam-1 cells (see Materials and Methods). The CHO/rRam-1 clones were then isolated by limiting dilution.

[b] Binding of amphotropic gp70 was measured in conditions of gp70 excess, using the antibody and [^{125}I]protein A method (see Fig. 2). The background of control CHO cells (1,300 cpm) was subtracted from the data shown. Errors are the range for duplicate assays.

[c] Measured by using the amphotropic virus that encodes human growth hormone and expressed as multiplicity of infection (MOI) as described in Table 1, footnote *b*. The differences in rRam-1 expression do not significantly correlate with infection efficiencies over this range of rRam-1 levels. Presumably, at lower rRam-1 levels the MOI would decline.

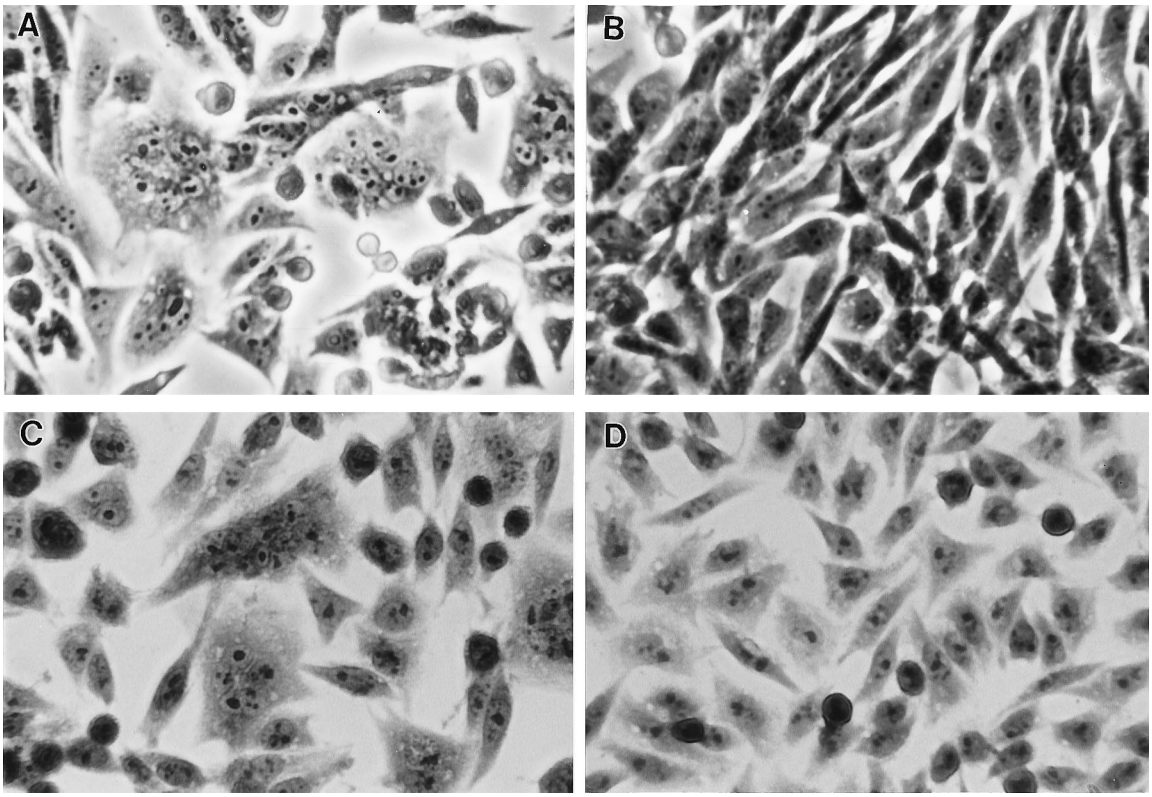

FIG. 7. Cytopathology caused by 4070A amphotropic virus infection of CHO/rRam-1 cells. CHO/rRam-1 cells infected with amphotropic 4070A show many multinucleated cells at 72 h postinfection (A), in contrast to uninfected controls (B). Cocultures formed by mixing 4070A-infected CHO cells with uninfected CHO/rRam-1 cells developed syncytia within 24 h (C), but these were absent when the 4070A-infected CHO cells were cocultured with CHO cells that lack rRam-1 (D). For panels C and D, CHO cells were infected with a pseudotyped 4070A virus as described in Materials and Methods, and amphotropic *env* glycoprotein expression was confirmed by immunofluorescence. The infected cells were seeded into culture plates and incubated overnight. The next day, CHO/rRam-1 cells or CHO cells were lifted with 8 mM EDTA in 0.9% saline, washed in complete medium, and added to the 4070A-infected CHO cell cultures. After 24 h of coculture, the plates were fixed in methanol and stained with toluidine blue (magnifications are ×590). Coculture results identical to those shown in panels C and D were obtained when the 4070A virus-infected cells were CHO/rRam-1 cells that had been allowed to recover from the cytopathic effects of virus infection; in all cocultures, syncytium formation required uninfected CHO/rRam-1 cells plus 4070A virus-infected cells.

rRam-1 perturbed processing of amphotropic MuLV 4070A *env* glycoproteins. This analysis was complicated by the cytopathology and recovery that occur in the cultures following virus infection (see above), and clear effects of rRam-1 on processing were not observed.

DISCUSSION

Role for rRam-1 in cell surface binding of amphotropic MuLV *env* glycoproteins. Our evidence supports the hypotheses that the gp70 envelope glycoprotein of amphotropic host range MuLVs binds to a cell surface pool of rat rRam-1 Na^+-phosphate symporter protein that is only slowly being endocytosed or degraded and that binding to the homologous hamster transporter is relatively weak. Adsorption of this gp70 onto CHO cells, as seen by three sensitive assays, is highly dependent on rRam-1 expression (Fig. 1 to 3). rRam-1 cDNA expression causes increased phosphate transport activity (Fig. 5) and adds novel relatively high-affinity gp70 binding sites to cell surfaces (Fig. 2). After attachment to these rRam-1-dependent sites, the gp70 remains accessible for many hours to antibodies or other reagents added to the culture media, and it appears to be lost from cell surfaces with a half-life of greater than 6 h (Fig. 4). Therefore, rRam-1 contributes to formation of a complex with amphotropic gp70 that has an extremely slow rate of dissociation. In agreement with these suggestions, expression of amphotropic *env* glycoprotein specifically down-modulates phosphate transport by rRam-1 but has no effect on the phosphate transporters of control CHO cells (Fig. 6). Extracellular amphotropic gp70 also seems to act as a weak specific inhibitor of the rRam-1 transporter (Fig. 6). Although these results are all consistent with direct binding of gp70 onto rRam-1, we emphasize that gp70-receptor complexes have not been identified or isolated and that rRam-1 might be merely a subunit or cofactor for the gp70 binding pathway. Moreover, the specific changes in the homologous hamster transporter that mask or otherwise prevent its strong interaction with amphotropic gp70 remain unknown. Nevertheless, our results establish that rRam-1 contributes to formation of relatively strong stable bonds with amphotropic MuLV *env* glycoproteins and not merely to postbinding steps of infection.

This investigation was initiated using a single clone of CHO cells (clone CERD-Cl9), thus reducing effects that might derive from clonal variation of these cells. As seen by immunofluorescence microscopy, none of these control CHO cells bound amphotropic gp70 to a detectable extent (e.g., Fig. 1), and similar results were obtained with other CHO cell lines that were analyzed (12). Moreover, all CHO/rRam-1 subclones that were derived from the CERD-Cl9 cells bound the gp70 strongly and were highly susceptible to infection and to cytopathic effects of amphotropic retroviruses.

Interference to superinfection. Amphotropic MuLV 4070A infection partially down-modulates the rRam-1 component of phosphate transport in CHO/rRam-1 cells (Fig. 6). Initially, this infection causes substantial cytopathology and cell-cell fusion (Fig. 7) and the apparent down-modulation can approach approximately 80% of the rRam-1 component of transport (Fig. 6A), whereas after more complete cytopathic recovery, the extent of down-modulation is reduced to only approximately 30 to 50% (Fig. 6B). Because these recovered cultures may have a slight residual cytopathology and because amphotropic gp70 may directly inhibit rRam-1 transporter activity (Fig. 6A), it is likely that 4070A virus infection may cause somewhat less than 35 to 50% removal of cell surface rRam-1. Similarly, human immunodeficiency virus type 1 (HIV-1) *env* glycoproteins partially down-modulate CD4, and additional HIV-1 genes (*vpu* and *nef*) are required for more complete CD4 removal (2, 8, 18, 42). Moreover, ecotropic MuLV infections partially down-modulate cell surface receptor expression (38). It has been suggested that gibbon ape leukemia virus infections strongly down-modulate Glvr-1 receptor expression, but the data would not exclude a partial loss of receptor (28).

Partial down-modulation of rRam-1 caused by amphotropic MuLV infection could not per se account for the complete interference to superinfection that occurs in these cells (Table 1) because CHO/rRam-1 clones with relatively little rRam-1 expression are infected as efficiently as clone 13 and clone 54 cells that have highest rRam-1 expression levels (Table 2). In agreement with previous evidence for other retroviruses (16, 39), these results suggest that almost complete removal of rRam-1 would be required to efficiently abrogate infection by a down-modulation mechanism. We infer that residual rRam-1 remaining on surfaces of 4070A virus-infected CHO/rRam-1 cells must be inactive as a viral receptor, probably because its virus binding site may be blocked by gp70. An important consequence of incomplete rRam-1 down-modulation would be the preservation of adequate phosphate uptake capability by infected cells that lack alternative phosphate transporter systems. Presumably, this would reduce cytopathology and benefit both virus and host.

Cytopathology by amphotropic MuLV 4070A. Infection of our CHO/rRam-1 cells with amphotropic MuLV 4070A initially causes a severe rRam-1-dependent cytopathology that involves a slowing of cell growth and extensive cell-cell fusions (Fig. 7). Our evidence strongly suggests that the fusion involves a coupling of infected cells that express amphotropic *env* glycoproteins with uninfected CHO/rRam-1 cells and that recovery occurs after all receptor-bearing cells become infected and the polyploid cells are substantially overgrown. Control CHO cells do not form syncytia in the same conditions (compare Fig. 7C and D). The reduced cytopathic effects that occur in control CHO cells infected with the same pseudotyped 4070A virus could involve weaker interactions of 4070A *env* glycoproteins with the homologous partially masked (25, 26, 43) hamster Ram-1 protein and/or changes that may not involve amphotropic *env* glycoproteins. Indeed, our preliminary studies suggest that the weak abnormalities that occur in our control CHO cell clone are caused by binding of the pseudotyped virus onto the cell surfaces and that they are independent of the 4070A viral genetic information (results not shown). The extensive cell-cell fusions caused by amphotropic virus 4070A infection of our CHO/rRam-1 cultures is compatible with other indications (22) that amphotropic MuLV membranes can fuse directly with surface membranes of susceptible cells. Our evidence that rRam-1 does not mediate rapid endocytosis of adsorbed gp70 (Fig. 4) also supports the idea that amphotropic virus may bind stably to rRam-1 and then infect by fusion of virion and cell surface membranes.

One of our assays, involving adsorption of gp70 onto cells followed by incubation with antibody to gp70 in the presence of complement resulted in specific killing of rRam-1-bearing CHO cells (Fig. 3). Presumably, such a process could result in immune-mediated killing of innocent bystander cells in infected hosts. In the case of HIV-1, lymphoid organs apparently contain nests of clonally infected cells that are surrounded by lymphocytes responsive to the specific viral antigens, and it is believed that these responsive lymphocytes may provide targets for infection and killing because of their immune activation and proximity to virus and to shed gp120 (5, 31). Presumably, such immune-mediated cell killing could be involved in depletion of virus-responsive lymphocytes and eventually in immunosuppression. Similar processes could be involved in the immune dysfunctions and cell killing that have been frequently associated with retroviral infections in mice and other species (27).

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